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13. ABSTRACT (Maximum 200 Words)
Nearly 20% of men who undergo a radical prostatectomy later relapse with bone metastases. The cellular events that are predictive of subsequent progressive disease remain unknown. We've focused attention on the detection of disseminated prostate cancer (CaP) cells in the blood and bone marrow. Our hypothesis is that these disseminated cells may provide critical insight regarding biomarkers of use in prognostication. We've developed enrichment and isolation techniques that allow the isolation of individual disseminated CaP cells for study as a pool of cells or single cells. Our proposal is to isolate these cells form 50 patients prior to radical prostatectomy and from 10 patients with advanced disease. The cells will be both phenotypically and molecularly analysed. To date 57 patients have been accrued and the analyses well underway. For example, we are finding that ~70% of patients prior to radical prostatectomy have disseminated CaP cells in their bone marrow. Also, we show a good correlation between patients with cytokeratin positive cells and human epithelial positive cells. In 36 of 37 specimens having disseminated CaP cells we found aberrant features of chromosome 8 by FISH analysis. Micro-array gene expression analysis is planned on single cells form the first 10 patients to recur.

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INTRODUCTION

One of the great challenges in the clinical management of prostate cancer (CaP) is determining the risk of progression in those patients thought to have localized disease. The recurrence rate among men who elect a radical prostatectomy for presumed localized disease ranges from 15 to 25%. Historically, two approaches have been taken to detect disseminated cancer cells and to improve the "staging" of patients at the time of diagnosis. In the first, microscopic scrutiny of disseminated cells in the blood of cancer patients by immunohistochemistry (IHC) and cytogenetic techniques revealed important information regarding the features of these disseminated cells in general (1-7). On a parallel path were efforts employing molecular technology. For example, ten years ago we (8) and Moreno (9) were the first to propose using the molecular technique of reverse-transcriptase-polymerase-chain-reaction (RT-PCR) to "molecularly stage" patients at diagnosis in hopes that detection of disseminated PSA+ cells in blood or bone marrow (BM) would be predictive of recurrence. Over time, efforts in this field showed that PSA RT-PCR positivity was not highly correlative with recurrence. However, we postulated that enrichment of the presumed CaP cells in the BM aspirate prior to RT-PCR testing might reveal a high pre-surgical detection rate and that techniques might then be developed to isolate the individual disseminated cells for study. We made use of magnetic particle cell enrichment techniques to study disseminated cells in CaP patients (10, 11, 16, 18). This approach provides for a much more robust analysis of the disseminated cell population, especially since the yield from BM aspirates can be several hundred to thousands of cells of interest. One of the most striking revelations in our series has been the documentation that ~55% of CaP patients prior to radical prostatectomy have disseminated cells in their BM aspirates following enrichment. The Objectives of this proposal center upon the study of these disseminated cells recovered from blood and BM and whether their biological features overall or as individual cells are predictors for progression. It appears logical that residual disseminated CaP cells in the BM (~90% of patients with advanced disease have bone metastases (12)) following a radical prostatectomy should provide at least as much insight as those associated with the primary tumor that is removed at surgery. The enriched population is adequate for microscopic analysis or for isolation of individual cells for further study. A powerful component of our proposed analyses involves cDNA micro-array-gene chips developed by our colleagues Leroy Hood and Peter Nelson. This technique combines the proven chemistry of nucleic acid hybridization with advanced automation and image analysis to quantitatively assess gene expression profiles (13-15). The expression levels of thousands to tens of thousands of genes represented at 0.01-0.001% abundance in a population can be simultaneously assessed. Thus our ongoing studies focus on generating molecular and IHC phenotypes of disseminated BM cells in fifty patients who are undergoing radical prostatectomy and having a Gleason Sum of 7 or greater. These profiles are contrasted to those of the primary tumor and to those in patients who have relapsed with bone metastases. A unique aspect of this proposal is our profiling of not only pools of disseminated cells but individual cells as the technologies advance to this level of sensitivity. Furthermore, comparisons of profiles will be made between these patient populations and a subset that demonstrates PSA biochemical relapse. These studies will provide the first multiparameter phenotypic analysis/discovery of potential progression markers that takes advantage of advances (a) in the recovery of disseminated CaP cells from blood and bone marrow, (b) in technology allowing the isolation of viable CaP cells to provide three degrees of heterogeneity (enriched, pooled homogeneous and individual cells) and (c) in the fields of cDNA microarrays and informatics that target small cell numbers

BODY

Hypothesis:

Disseminated CaP cells isolated from the blood and BM at the time of radical prostatectomy will reveal biological features useful in assessing the probability of relapse. Furthermore, the analysis of single cells isolated from the enriched population will provide a second level of discrimination reflective of the heterogeneity of disseminated cells and allow detection of rare, but important, features not revealed in pooled, groups of disseminated cells.

Technical Objectives:

Task #1: Define the predominant phenotype(s) of prostate cancer bone metastases

- Identify 10 patients with advanced prostate cancer involving the bone who have had a radical prostatectomy and were found to have a Gleason Sum $\geq$ 7. Following informed consent, obtain bone marrow aspirates. Using our para-magnetic enrichment techniques, we will derive an enriched population of disseminated CaP cells. From this population, we will "pluck" individual CaP cells and pool into sets of 50-100. These are then phenotyped using RT-PCR, PCR, FISH, ISH and micro-arrays (micro-array core facility of Peter Nelson, proposal co-investigator).
  (0 – 12 months)

Task #2: Establish phenotype(s) of primary tumor, and disseminated cells in blood and bone marrow by enrichment and pooling of cells from 50 patients undergoing prostatectomy and having Gleason Sum $\geq$7.

- Under separate funding we routinely obtain blood and bone marrow aspirates from all consenting patients prior to radical prostatetomy. From this large population this proposal involves a subset that consists of patients who are found to have a Gleason Sum $\geq$ 7. We will select 50 of these patients for the studies herein. Using our para-magnetic enrichment and isolation techniques, we will derive populations of disseminated CaP cells in three degrees of "purity": (a) enriched, (b) isolated individual cells that are pooled (50-100 cells) and (c) individual cells (20+). Using the phenotyping protocols of Task #1, sets (a) and (b) will be phenotyped from blood and from bone marrow. Set (c) will be partially purified and stored at –80° C for use in Task #3. We will also obtain at least 2 primary tumor foci (microdissected) for phenotyping.
  (0 – 24 months)

Task #3: Establish the phenotype of individual disseminated cells following enrichment and isolation from the bone marrow in patients of Aim #2 who experience biochemical (PSA) failure.

- Among the cohort of 50 patients, monitor for PSA biochemical recurrence using our ultrasensitive PSA chemiluminescent assay with which we've reported confidence in detecting relapse at a PSA serum level of 0.05 ng/mL.
  (0-30 months)

- Identify the first 10 patients who experience a biochemical recurrence and 10 who do not show any evidence of recurrence. Retrieve from –80°C storage the individual aliquots of disseminated CaP cells (bone marrow derived only) that were processed in Task #2. Phenotype, primarily by micro-array, these 400 individual CaP cells (20 cells/patient x 20 patients [10 fail + 10 no fail]). (20 – 36 months)

- Compare and contrast all data sets, perform statistical analyses. (30 – 36 months)

Results:

This past year saw exceptional progress in some areas and slower progress in others. As noted in last year's report, we were delayed by nine months in year 1, in our plan to accrue patients into the study because of a disagreement between the University of Washington IRB and the DoD over language in the consent form. A total of 60 patients were to be accrued, consisting of two patient populations, prior to radical prostatectomy and advanced disease. I am very pleased to reveal that as of the writing of this report, we've accrued 54 pre-radical prostatectomy patients and 3 advanced stage patients.

I also mentioned in last year's report that with non-DoD funding, we were acquiring a better fluorescent microscope with which we would be more accurate with the detection of the disseminated cancer cells and the performance of fluorescent dependent phenotypic assays. However, what we initially found, was that there became a distinction among two populations of enriched epithelial cells from the bone marrow aspirates which had not been noticed with the older microscope. Our procedure uses negative (CD45 and CD61) selection followed by positive selection (anti-human epithelial antigen; HEA). The positively selected epithelial cells are detected under fluorescence using a FITC-labeled anti-HEA antibody to a different epitope than the antibody used for selection. The detected cells are then retrieved (i.e. "plucked") using a micromanipulator and pipette system attached to the inverted fluorescent microscope. In addition to the vast majority of non-fluorescent cells we noted now the two populations of fluorescent cells. One population was quite a bit more lightly stained than the other. The lightly stained population was in excess. We spent a considerable effort in trying to resolve this issue. The brightly stained population appeared to be the disseminated prostate cancer cells by morphology and molecular analysis whereas the lightly stained population appeared to be cells of other non-epithelial origin. An extensive literature search revealed one paper by Lammers et al (19) which stated that the human epithelial antigen resides in low abundance on a pre-erythroid stem cell population. Follow-up phone calls to other investigators using anti-HEA and to the commercial sources of anti-HEA antibodies failed to derive supportive evidence of this work. However, because the selective "plucking" of these cells followed by RT-PCR failed frequently to reveal a PSA message, we decided to retrieve only the more brightly stained cells for study. The consequence of this decision was that far fewer cells were obtained than with the other older microscope which did not distinguish among the two populations. Because of this, we have been forced to reduce the number of descriptive phenotypic studies in some patient samples in preference to storing the most positive cells for micro-array analysis in year 3. This has especially impacted the peripheral blood specimens where we have preferentially stored the

few positive cells per sample for future single cell micro-array studies rather than attempt phenotypic studies on just a very few cells. The percentage of patients prior to radical prostatectomy with PSA positive disseminated cells did not change appreciably in these 54 pre-radical prostatectomy patients, i.e. ~30 % in the peripheral blood and ~70% in the bone marrow. Pools of positive cells and sets of individual positive cells have been obtained on all patients accrued to date where disseminated cells were detected. We intend to far exceed our accrual goal. The radical prostatectomy patients are being monitored for biochemical failure as part of Task 3.

On all of the patients where we acquired a peripheral blood specimen and bone marrow aspirate prior to surgery we also obtained a tumor specimen at radical prostatectomy. These tumor specimens were embedded in OCT and flash frozen. As part of Task 2, two foci from each specimen in patients yielding a sufficient number of disseminated CaP cells for analysis, will be microdissected for comparison to the results from the disseminated cells. While we did not stipulate the method of microdissection in the proposal we have subsequently decided, based on our experiences from other funded projects, that laser capture microdissection is the preferred approach. At approximately month 8 into year 2, we were successful in recruiting a technician who would focus on this aspect of the proposal. Recruitment had been delayed since so few patients had been accrued by early year 2. This laser capture microdissection and phenotyping work is proceeding well.

The ability to perform micro-array analysis on less than several hundred cells, let alone pools of approximately 20 cells or single cells as we proposed, has been an excrutiating technical challenge to those in the field. We had hoped to report that this challenge has been resolved and that analysis of the stored disseminated cells from our patients had begun. While this is not yet the case, we are pleased that significant progress is being made and those who are attempting to resolve this sensitivity issue are confident that a procedure will be available shortly. During the past year, Pete Nelson, M.D. who is our collaborator at the FHCRC and his team have worked on this challenge. For example, we provided sets of LNCaP cells containing from 10 to 2500 cells to test the feasibility of two rounds of linear amplification followed by quantitative RT-PCR analysis. Up to 70 ug of aRNA was obtained from 10 LNCaP cells. Figures 1 and 2 demonstrate the success of these studies. In Figure 1, we demonstrate that the threshold cycle increases as the cell count decreases per unit aRNA reproducibly between sets; the difference between the samples with highest and lowest cell number is only 5-6 cycles. Figure 2 shows that throughout this range the fold difference is quite reproducible among samples, differing by less than 2 fold.

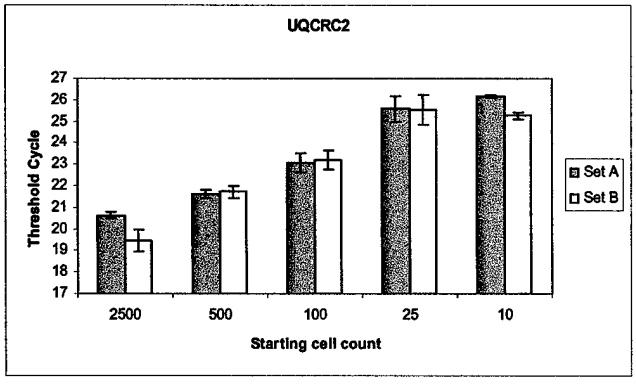

Figure 1

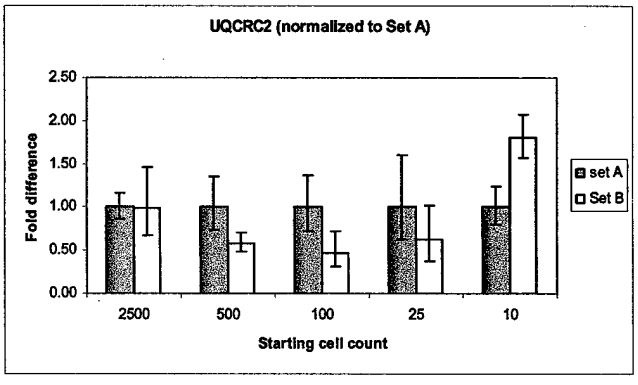

Figure 2

Progress has also been made in the phenotypic analysis of the disseminated cell population from the BM aspirates. Using a cytokeratin stain, we obtained almost identical percentages of patients positive for cytokeratin positivity and HEA positivity (e.g. ~70% in BM). In the study of double-staining for cytokeratin and Ki-67, only 15% of the patients with cytokeratin positive cells also revealed Ki-67 stained cells. Within this small population, the percentage of cytokeratin positive cells that were Ki-67 positive was less than 10%. Thus, very few of the disseminated prostate cancer cells in the pre-radical prostatectomy population are actively undergoing division. A rare example of a Ki-67 positive cell (also cytokeratin positive) obtained from a bone marrow aspirate is shown as Figure 3.

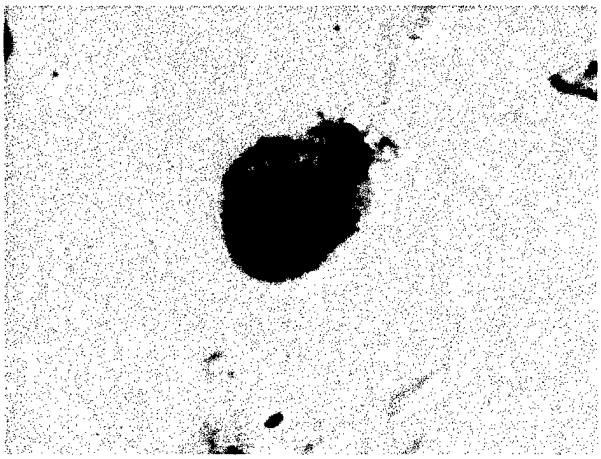

Figure 3

One interesting notation that conforms to our previous observations is that approximately a third of the population with HEA positive and/or cytokeratin positive cells do not have PSA staining cells. This implies that these cells, once separated from the supportive stroma, stop producing PSA at levels necessary for immunohistochemistry detection.

All of the bone marrow specimens having cytokeratin positive cells (~70% per above), have been analyzed by FISH (Vysis ProVysion). This multiprobe kit for chromosome 8 allows for detection of increase or decrease of chromosome 8 and deletion/loss of 8p/8q. The detailed analysis is underway but in 36 of the 37 specimens, chromosome 8 aberrations were noted. This is further evidence that malignant cells comprise at least a segment of the disseminated cell population. Figure 4 shows a disseminated, cytokeratin positive cell (A) with 2 copies of chromosome 8 and 8q with one copy (red) of 8p (B).

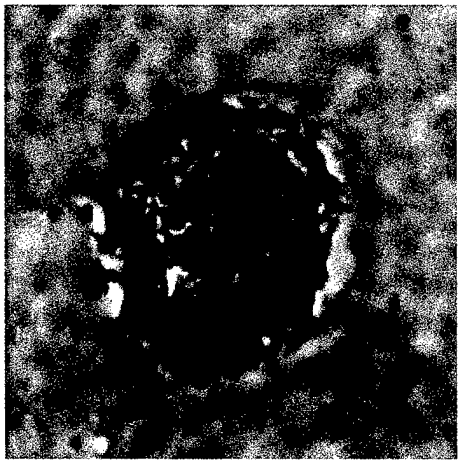

Figure 4A

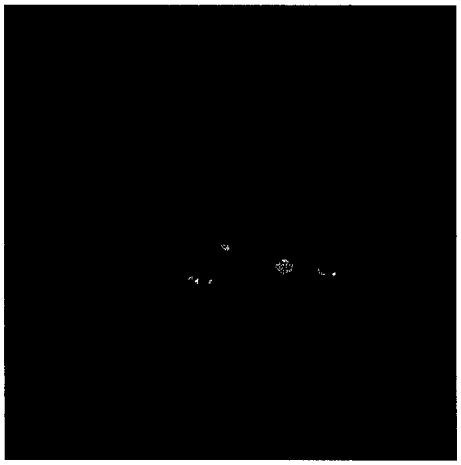

Figure 4B

For the RT-PCR analysis of ~20 individually "plucked" and then pooled cells from the enriched bone marrow aspirates, 26 of the 37 disseminated cell positive specimens had a sufficient number of cells for the pooled studies. As expected, ~50% of these pooled cell sets were PSA RT-PCR positive, and nearly 90% were epithelial glycoprotein RT-PCR positive.

Despite numerous combinations, we have been unable to find a set of primers for Prostate Stem Cell Antigen (PSCA) that does not give a positive reaction to normal bone marrow cells. Therefore, until this can be resolved, we are substituting real-time EZH2 RT-PCR for PSCA. This work has just begun but of pooled cell specimens from 10 patients, 3 were positive. EXH2 has been associated with prostate cancer disease recurrence (17).

KEY RESEARCH ACCOMPLISHMENTS

- Following a very slow start of patient accrual in year 1 due to IRB administrative issues, we have now enrolled 57 of the 60 patients and are on schedule. Due to low disseminated cell numbers in some of these patients we will increase the accrual number significantly of the pre-radical prostatectomy group. We already plan on asking for a no-cost, one year extension to allow time for biochemical recurrences to occur in 10 patients.

- We've made extensive progress in the phenotypic analysis of the disseminated cells from the bone marrow aspirates using the markers PSA, cytokeratin and Ki-67 and in FISH analysis of chromosome 8. The GSTpi studies have just begun.

- Good progress has been made in assessing specific gene expression profiles of isolated pools of 20 individual cells by RT-PCR or real-time RT-PCR including PSA, PSMA, EGP and EZH2. AR methylation studies have begun in those patients where sufficient disseminated cells are available but this is of lower priority.

- Despite intensive efforts, we are still not comfortable with gene expression microarray analysis using a single cell or even a pool of 20 individual cells. These pools of single disseminated cells and the individual cells have been "plucked" and stored from the patient samples but because they are so valuable, we won't engage in the microarray analysis until the procedure is more robust. This is a huge technical challenge, as are many of the aspects of this proposal. Slow but steady progress is being made by our collaborator Peter Nelson in reaching the goal of using few to single cells for this task.

REPORTABLE OUTCOMES

Abstracts:

Pfitzenmaier J, Arfman E, Klein J, Winch R, Nance M, Lange P and Vessella RL. New enrichment method for the isolation and characterization of circulating prostate cancer cells (CPCC) from the peripheral blood (PB). Proc. Am Assoc. Cancer Res 43:433 #3635

Pfitzenmaier J, Ellis WJ, Arfman E, Klein JR, Lange PH and Vessella RL. A method to isolate disseminated prostate epithelial cells and the comparison of their detection rate to standard RT-PCR. Proc. Am Assoc. Cancer Res 44:42, #216

Pfitzenmaier J, Ellis WJ, Arfman EW, McLaughlin PO, Lange PH and Vessella RL. Telomerase activity in circulating prostate cancer cells. Proc. Am Assoc. Cancer Res 44:42, #5193

CONCLUSIONS

Our year 2 progress is a great contrast to the slow start in year 1 due to the nine month delay in patient accrual due to DoD IRB issues. We have accrued nearly all of the patients proposed but intend to continue and expand the accrual numbers. We have processed all of the peripheral blood and bone marrow aspirates, enriching for disseminated prostate cancer cells. On those specimens with evidence of disseminated prostate cancer cells we have made extensive progress in both the phenotypic and molecular characterization of these cells. Also aliquots of individual cells have been "plucked" and stored until there is evidence of biochemical recurrence in at least 10 radical prostatectomy patients. While several technical challenges remain ahead of us, significant progress is being made. To our knowledge, we still remain the only group applying the most advanced micro-array expression technologies with those of classical phenotyping and FISH analysis to the study of disseminated prostate cancer cells isolated from the bone marrow down to the single cell level.

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